(11)Pub.# 08269088
(43)Date of pub:10/15/1996
(51)Int.Cl. c07k 7/06
a61k 38/55
a61k 38/55
(21)Appli. #07070156
(22)Date of filing :03/28/1995
(71)Applicant: Snow Brand Milk Prod Co Ltd
(72)Inventor: Hisatoshi Ito, Tadao Saito, Yutaka Wakao, Toshiaki Uchida, Kiyoshi Tatsumi.

(54)New Peptide and Its Utilization
(57)Abstract:

Purpose: To obtain a new peptide having an angiotensin converting enzyme-inhibiting activity and useful for antihypertensives, health foods, cosmetics, animal feeds, etc., by separating and purifying the peptide from the protease decomposition product of a bovine K-casein glycomacropeptide.
Constitution: Anew peptide having an amino acid sequence of the formula. Since the peptide has an angiotensin converting enzyme-inhibiting activity and exhibits an antihypertensive action, the peptide is added to various medicines such as antihypertensives in the form of injections, sugar coated tablets, tablets or capsules, beverages or foods, refreshing beverages, fruit juices, fermented beverages, jellies r ice creams, and is useful for health foods used for treating and preventing hypertension, for cosmetics for imparting a vasodilating action, for animal feed additives, etc. The peptide is obtained by separating a K-casein glycomacropeptide from a whey protein concentrate obtained from milk, treating the separated K-casein glycomacropeptide with a protease such as pepsin, and subsequently subjecting the decomposition products to a gradient elution treatment with a column chromatography, etc., to fractionate the decomposition products.
Ile-Ala-Ser-Gly-Glu-Pro (I)

(Range of Patent Petition)
(Petition1)Amino acid sequence: Ile-Ala-Ser-Gly-Glu-Pro
(Petition2) Angiotensin converting enzyme (ACE)-inhibitor including Ile-Ala-Ser-Gly-Glu-Pro
(Petition3)Antihypertensive medicine including Ile-Ala-Ser-Gly-Glu-Pro
(0001)
(Extent of commercial use)
This invention is about angiotensin converting enzyme-inhibitor from new peptide, and about antihypertension medicine.
(0002)
(Former technique)
ACE affects in Renin-angiotensin system (r-a) about adjusting blood pressure. Angiotensinagen, MW about 57,000, changes to angiotensinI when it reacts with renin from kidney. . When angiotensinI reacts with ACE, it produces angiotensinII that contracts peripheral blood vessel (Furchgott, R. et al., J.PharmacolExp. Ther., vol. 108, p.129, 1953), increased blood vessel osmosis (Gimbrone, M.A. and Alexander, R.W., Science, vol.31 p.189, p219, 1975), promoting adrenergic neurotransmission in sympathetic nerve (Bell, C., Circ.Res., vol.31, p.348, 1972), and increasing catecholamine from adrenal medulla and promotes aldosterone from adrenal cortex (Biron, P. et al., J.Clin. Invest, vol.44, p.1171, 1965). They increase blood circulation to increase blood pressure. ACE breaks bradykinin that decreases blood pressure.
On the other hand, ACE inhibitor stops ACE reaction to decrease blood pressure.
(0003)
Because it is paid attention more that increasing life-style and aging related diseases in society, There are antihypertension medicines today. However, preventing hypertension still relies on diet. People expect to have antihypertension added into food ingredients.

(0004)ACE inhibitor in food
-Hydrolyzed protein from sardine muscles (Nihon Fishery, Inc.)
-ACE inhibitor from krill by ion exchange, gel filtration, and reverse chromatography (Y. Kawamura, micronutrient research, vol. 7, p.37, 1990).
-oligopeptide from corn protein(pub.#02240027).
(0005)Peptides from milk protein casein have high content of ACE inhibitor.
-Peptide from amino acid sequence 39~52 residue of human β casein(Kohmura, M. et al., Agric.Biol. chem., vol.53, p.2107,1989).
-Peptide from amino acid sequence 43~52 residue of human κ casein(Kohmura, M. et al., Agric.Biol. chem., vol.54, p.835,1990).
-Peptide from amino acid sequence 23~34 and 194~199 residue of bovine αs1 casein (Maruyama, s. et al., Agric. Biol. Chem., vol.51, p.2557, 1987).
- Peptide from amino acid sequence177~183 residue of bovine β casein (Maruyama, s. et al., Agric. Biol. Chem., vol.51, p.1581, 1987).

-Peptide from amino acid sequence 25~34 residue of bovine κ casein (Tozuka, M. Uenokawa, S. Japan livestock newsletter, vol.63, p.867, 1992).
-ACE inhibitor from bovine α and β casein prevents hypertension (Pub.#02167052)
-bovine κ-casein glycomacropeptide from amino acid sequence after 106 residue suppresses increasing blood pressure (Pub.#06345664).
(0006)
(Object of the invention)
The inventors find the new ACE inhibiting peptide from hydrolyzed bovine κ-casein glycomacropeptide in cheese whey. They confirm that the peptide decreases blood pressure of animals. Their object of invention is to provide ACE inhibitor that decreases blood pressure.
(0007)
(Methods)
The inventors find the new ACE inhibiting peptide from hydrolyzed bovine κ-casein glycomacropeptide in sweet cheese whey. They confirm that the peptide decreases blood pressure of animals. Their object of invention is to provide ACE inhibitor that decreases blood pressure.
Ile-Ala-Ser-Gly-Glu-Pro (I)
(0008)The new peptide of amino acid sequence
-20~25 residue of κ-casein glycomacropeptide.
-125~130 residue of κ-casein.
(0009) The inventors confirm that the peptide, Ile-Ala-Ser-Gly-Glu-Pro, decreases blood pressure
(0010)Although the pure new peptide can be used for food, cosmetics, animal foods, and medicine, low purity ones still remain the strength of ACE inhibiting activity of pure one. It can be useful after hydrolyzed by enzyme from κ-casein glycomacropeptide or papain. It can be synthesized by solid method with Applied biosystem. The peptide can be produced by DNA synthesis, because the nucleotide sequence of bovine κ-casein has been revealed (Alexander, L.J. et al., Eur.L.Biochem., vol178, p.395,1988). The nucleotide sequence, ATT GCT AGT GGT GAG CCT, is what needed to synthesize the new peptide.
(0011)The procedure
- Extracting κ-casein glycomacropeptide
Use one of any methods mentioned below:

1. -dissolve cheese whey powder with distilled water, and heat it.
   -Cool it, and add some ethanol to it.
   -Collect the supernatant, and use anion exchange resin to extract κ-casein glycomacropeptide (Saito,T. et al., J.Dairy Sci., vol.74, p.2831, 1991).
2. -Adjust pH of the by-product from rennet casein curd, and collect the supernatant.
   -Desalting the supernatant to extract κ-casein glycomacropeptide (Pub.#63284199).
3. -Adjust pH of milk ingredient material to less than 4.0, and filter with a membrane, MWcut-off 10,000~50,000.
   -Ultra filtrate, MW cut-off less than 50,000, to concentrate κ-casein glycomacropeptide (pub.#02276542).
4. -Heat whey protein liquid, and freeze it.
   -Thaw it, and separate whey protein and supernatant (Pub.#03294299).

(0012)Hydrolyzing κ-casein glycomacropeptide
-Use pepsin preferably, but it is possible to use papain or acid.
-The concentration of pepsin is 1/10~1/10000 (w/w), and temp. is 30~40C.
-PH is preferably between 1~4.
-The buffers: citric acid, acetic acid, KCl-HCl, (glycine+NaCl)-HCl.
-Also use HCl solution to adjust pH .
-The reaction time is more than 10 min., preferably 1~48 hours.
-If less than 10min., ACE inhibiting activity is less than 20%.
-If more than 48 hours, start contamination and weak enzyme activity.
-More pepsin can be added later.
-Heat at 100C for 5 min to stop enzyme reaction, and also use cool acetone or NaOh to adjust pH 7.0.
-Remove the precipitation, and concentrate it by vacuum, freeze, and spray dry, and reverse osmosis, electro dialysis, and film evaporator if necessary.
-ACE inhibiting activity is 10~100μg/ml.
-It is safe as food ingredient.
-Poison test result shows that this peptide is more than 1g/1kg by oral administration.
(0013)The peptide can be added to foods, cosmetics, animal feeds, and medicine as the form of liquid, powder, or tablets.
-It is safe because it is from milk.
-The amount of dose is more than 150μg/kg/day to activate the antihypertension activity better.
(0014)
(Example1)
-Add trichloroacetic acid solution to 150g of cheese whey powder to adjust concentration of 12%(w/v).
-Heat at4C for 1hour, and centrifuge at 9,000Xg for 15min.
-Collect supernatant, and cool down.
-Neutralize with 1NnaOh, and do osmosis with membrane and distilled water.
-Centrifuge at 9,000Xg for15min., collect supernatant, and do freeze dry to make 7g of purity 81% of κ-casein glycomacropeptide.
(0015)
(Test1)Test of digestion of κ-casein glycomacropeptide from example 1 with 5 different proteinase.
(1)ProteinaseK(Sigma) and 0.01M tris-HCl buffer(pH7.5).
(2)ActinaseE(Nakaraitesk) and 20mM of 0.1Mtris-HCl buffer, including CaCl(pH7.4).
(3)Pepsin(Sigma) and 0.2M trichloroacetic acid buffer(pH3.0).
(4)Trypesin (sigma)and 20mM of 0.1Mtris-HCl buffer, including CaCl(pH8.0).
(5)Papain (Sigma) and 0.1 phosphate buffer(pH7.0), including 3.3mg of sodium cyanide and EDTA.
(0016)-Mix 10mg of κ-casein glycomacropeptide with each 10ml of the buffer, and add 5%(w/w) of concentration of enzyme.
-Digest it with small amount of toluene at 37C for 24 hours, and add cool acetone 3 times of the amount of the solution to stop the reaction at -20C for 1 hour.
-Filtrate, and do freeze dry.
-Mix 1mg with 200μl of distilled water, and dilute 15μl of the solution with 150μl of distilled water.
-Measure ACE inhibiting activity.
(0017)
(Table1)the result

Enzyme	ACE inhibiting activity(%)
(1)	0
(2)	0
(3)	97
(4)	0
(5)	5
κ-casein glycomacropeptide	0

(0018)The method of measurement of ACE inhibiting activity
-ACE solution (A):
Mix 1 unit of ACE(rabbit lung, Wako Pure Pharmaceutical Industry) with 2ml of 50% glycerol solution.

-Substrate solution(B)
Mix 53.688mg of hippuric acid-His-Leu with10ml of boric acid buffer(pH7.8), including 1M NaCl, and adjust pH8.3 with NaOh.
(0019)-Mix 150μl of enzyme solution with 100μl of (B) and 10μl of (A) in a test tube, and heat at 37C for 60min.
-Add 1.5ml of ethyl acetate, and mix it by using a vortex mixer for 15sec. to stop the reaction.
-Centrifuge at 1000rpm for 5min., and Collect supernatant.
-Spray dry in a small tube with nitrogen gas at 80C, and heat at 80C for 5min.
-Cool down, add 3.0ml of NaCl solution, and mix it for 15sec. with a vortex mixer.
-Measure absorption at 228nm.
-ACE inhibiting activity(%)={(Ec-Es)/(Ec-Eb)}X100
-Es: Absorption of the sample with 150μl of enzyme solution.
-Ec: Absorption of the sample with 150μl of distilled water.
-Eb: Absorption of the sample with 150μl of enzyme solution right after stopping the reaction.
(0020)Because of the result shows that pepsin is the strongest activity, the inventors start looking for a sample that contains ACE inhibitor with pepsin.
(0021)
(Example2)Making hydrolyzed κ-casein glycomacropeptide sample by pepsin.
- Mix 10mg of κ-casein glycomacropeptide with 10ml of 0.1NHCl solution, and add 5%(w/w) of concentration of pepsin.
-Digest it with small amount of toluene at 37C for 24 hours, and heat at 100C for 5min. to stop the reaction.
-Filtrate, and do freeze dry.
(0022)-Clean 10ml of Butsirutoyopearl(Toso, Inc.) with 90% methanol, rinse with distilled water, and fill it into a column(1.7cmX12cm).
-Make the solution, concentration 10mg/5ml of the sample(example2), and run it through the column.
-Run 200ml of distilled water, and extract the elutes by using 100ml of 10 % different concentration from 10 to 100%.
-Concentrate each elute by vacuum, and do freeze dry.
-Measure ACE inhibiting activity by the test1 method.
-The activity is 70%.
(0023)
(Example 3)-Mix 9mg of the powder sample (example2) with 5ml of distilled water, and run it through the column(1.7cmX12cm) that contains 10 g of RecropulepRP18(Melk) after cleaning with 90% methanol and rinsing with distilled water.
-Run 200ml of distilled water, and extract the elutes by using 100ml of 10 % different concentration from 10 to 100%.
-Concentrate each elute by vacuum, and do freeze dry.
-Measure ACE inhibiting activity by the test1 method.
-The activity is 90% in 10~20% methanol concentration elute.
-The activity is 15~50% in 30~60% methanol concentration elute.
-Collect the elute of 10~60% methanol concentration.
(0024)
(Example4) -Mix 7mg of the powder sample (example3) with 5ml of distilled water, and run it through the column(1.7cmX12cm) that contains 10 g of RecropulepRP18(Melk) after cleaning with 90% methanol and rinsing with distilled water.
-Run 200ml of distilled water, and extract the elutes by using 100ml of 5, 10, 12, 14, 16, 18, 20, 30, and 90% of different concentration.
-Concentrate each elute by vacuum, and do freeze dry.
-Measure ACE inhibiting activity by the test1 method.
-The activity is high in 5 and10% methanol concentration elutes.
(0025)
(Example5)-Purify the sample (example4) with the column(ODP-50, 7.6X250mm, Asahipack) after equilibrating with 10% acetonitrile solution(A), including 0.05% trifluoroacetic acid.
-Extract the elute by using gradient method of 60% acetonitrile solution(B), including 0.05% trifluoroacetic acid, and adjust (B) to 50% of acetonitrile solution after 30min.

-The flow is 0.5ml/min.
-Collect the elute that high in ACE inhibiting activity between 10~ 18min. of the retention time.
(0026)
(Example6)-Purify the sample (example5) with the column(Superiorex ODS, 4.6X150mm, Shiseido) after equilibrating with 0.05% trifluoroacetic acid solution(A).
-Extract the elute by using gradient method of 60% acetonitrile solution(B), including 0.05% trifluoroacetic acid, and adjust (B) to 50% of acetonitrile solution.
-The flow is 0.5ml/min.
-The result with HPLC is on Figure1.
(0027)
(Example7)-Find amino acid sequence of the peak from Figure1with amino acid sequencer(473A, appliedbiosystem), and analyze the sequence after dot plotting 1μg of the sample on PVDF membrane.
-The sequence is Ile-Ala-Ser-Gly-Glu-Pro.
(0028)
(Example8)To prove ACE inhibiting activity of the sample(example7), synthesize the peptide.
-Adsorb the peptide by t-Moc method with peptide synthesizer (430A, Appliedbiosystem) filled with 0.5mmol Boc-l-Cln-O-CH2-PAM resin and 2mmolamino acid.
-Desorb the peptide from 1.5g of the resin by using trifluoromethanesulfonic acid, including thioanisole and ethanedithiol.
-Precipitate with diethyl ether, and dissolve in 10% acetic acid.
-Purify with strong acid base anion ion exchange resin(Bio-Rex MSZ 1-X8), and collect 140mg of the peptide.
-Run it through reverse HPLC of Aquapac RP-300(Appliedbiosystems) with 0.1% trifluoroacetic acid-water solution and 0.1% trifluoroacetic acid- acetonitrile.
-Collect 30mg of white powder, and measure ACE inhibiting activity by the test1 method.
-The result is on Table2.
(0029)
(Table2)

Peptide	ACE inhibiting activity(%)
Peptide of Ile-Ala-Ser-Gly-Glu-Pro(example6)	100
Peptide of Ile-Ala-Ser-Gly-Glu-Pro(example7)	97

(0030)
(Test2)Effect on treating hypertension with the peptide, Ile-Ala-Ser-Gly-Glu-Pro (I).
-Animal test with 4 weeks old natural hypertension SHR/Ncri rat(Nihon Charles River).
Group A: Feed (I) 300μg/day.
Group B: Feed (I) 30μg/day.
Group C: Feed (I) 3μg/day.
Group D: Feed (I) 0μg/day.
(0031)-One group is 5 rats.
-Feed CE-2(Crea) that no casein twice a day by forcedly for 6 weeks, and no limit for water.
(0032)
(Table3)

Group	Beginning	After 6 weeks
A	126±7	203±7
B	125±4	232±5
C	130±5	246±9
D	128±6	259±8

-Numbers: Avg±Div.
-Group A and B show lower blood pressure.
(0033)
(Practice1) -Produce 50g of κ-casein glycomacropeptide from 1kg of whey protein concentrate (WPC) by the method of Pub. #02276542.
-Mix this with 1250g of water, and adjust pH 1.5 with HCl.

-Add 0.5g of pepsin(Sigma), and heat at 37C for 15 hours to react enzyme activity.
-Stop the activity by heating up to 100C for 5 min, and filter with filter paper (Advantech).
-Do freeze dry, and collect 37g of the sample.
-dissolve this 37g into 1850g of distilled water, and run with the column(20cmX20cm) that contains 5kg of Biocill C18HL(Bio-Rad) after equilibrating with distilled water.
-Desorb with 60% methanol solution after wash with distilled water, and vacuum concentrate it.
-Do freeze dry, and collect 12g of the sample.
-Mix 300mg of the sample with 1ml of 2% acetonitrile solution, including 0.1% trifluoroacetic acid.
-Run through the column(TSKgel ODS-120T, 55mmX60cm, Toso) after equilibrating with 2% acetonitrile solution, including 0.1% trifluoroacetic acid, and desorb with 50% acetonitrile solution, including 0.1% trifluoroacetic acid by gradient method.
-Collect the elute when acetonitrile concentration is 10% by measuring with HPLC.
-Repeat this, concentrate with a centrifuge, and do freeze dry.
-Collect 1.8g of the sample.
(0034)-Measure the purity of the sample with Superiorex ODS (4.6X150mm, Shiseido).
-The purity is 93%, and amino acid sequence is Ile-Ala-Ser-Gly-Glu-Pro (I).
-The inventors name the peptide "κ Caseinosin".
(0035)
(Practice2)-Adsorb the peptide by t-Moc method with peptide synthesizer (430A, Appliedbiosystem) filled with 0.5mmol Boc-l-Cln-O-CH2-PAM resin and 2mmolamino acid.
-Desorb the peptide from 1.5g of the resin by using trifluoromethanesulfonic acid, including thioanisole and ethanedithiol.
-Precipitate with diethyl ether, and dissolve in 10% acetic acid.
-Purify with strong acid base anion ion exchange resin(Bio-Rex MSZ 1-X8), and collect 140mg of the peptide.
-Run it through reverse HPLC of Aquapac RP-300(Appliedbiosystems) with 0.1% trifluoroacetic acid-water solution and 0.1% trifluoroacetic acid- acetonitrile.
-Collect 30mg of white powder, and measure amino acid sequence.
-The amino acid sequence is Ile-Ala-Ser-Gly-Glu-Pro (I).
(0036)
(Practice3)From Saito(J.Dairy Sci., vol.74, p.2831, 1991), Produce 13g of κ-casein glycomacropeptide from 1kg of cheese whey powder.
-Mix this with 0.33L of citric acid buffer(pH3.0).
-Add 0.01g of pepsin(Sigma), and heat at 37C for 48 hours to react enzyme activity.
-Stop the activity by adding 1.95L of cool acetone , and concentrate to 0.5L with a evaporator.
-Do freeze dry, and collect 11g of the sample.
-The ACE inhibiting activity is 100% in 100μg/ml by the method from test1.
(0037)
(Practice4) Produce 17g of κ-casein glycomacropeptide from 1kg of cheese whey powder by the method of Pub. #02276542.
-Mix this with 65L of 0.1Mtris-HCl buffer(pH7.5).
-Add 0.17g of papain , and heat at 37C for 20 hours to react enzyme activity.
-Stop the activity by adding 1.95L of cool acetone, and concentrate to 0.5L with a evaporator.
-Do freeze dry, and collect 15g of the sample.
-The ACE inhibiting activity is 9% in 100μg/ml by the method from test1.
(0039)
(Practice5)-Produce a test beverage with the peptide from Practice1 for preventing hypertension.
-It contains: 150mg the peptide, 3.2g of sucrose, 53g of citric acid, 53g of sodium citric acid, 0.06g ofvitamineB2, 0.37g of vitamin, 0.03g of folic acid, 6.7g of flavoring agent, 134g of 5X apple juice, 3.5kg of water.
-Heat the mixer at 90C for 10sec., and cool down to 5~10C.
-Fill 350ml of the liquid into a sterilized bottle.
(0039)
(Practice6) Produce a test tablet with the peptide from Practice2 for inhibiting hypertension.
-It contains: 20mg of the peptide, 33.3mg of lactose, 16.4mg of corn starch, 12.8mg of carboxymethyl cellulose calcium, 1.5mg of stearic acid magnesium.

(0040)
(practice7)-Produce a test animal feed with the peptide from Practice3.
-It contains: 1g of the peptide, 60g of non fat dry milk, 14.3g of WPC, 17.2g of fat, 5g of glucose, 2.5g of vitamins, 2.5g of minerals.
(0041)
(practice8))-Produce a test infant milk with the peptide from Practice1 for preventing hypertension.
-It contains: 750mg of the peptide, 1,195g of non fat dry milk, 263g of whey powder, 119g of vegetable fat, 5g of vitamins and minerals.
(0042)
(Effect of invention)This peptide is very safe because it is from milk. The peptide is added to various medicines such as antihypertensives in the form of injections, sugar coated tablets, tablets or capsules, beverages or foods, refreshing beverages, fruit juices, fermented beverages, jellies r ice creams, and is useful for health foods used for treating and preventing hypertension, for cosmetics for imparting a vasodilating action, for animal feed additives, etc.

(Figure 1)